Applied and Environmental Microbiology, Feb. 2011, p. 1405–1412 Vol. 77, No. 4
0099-2240/11/$12.00 doi:10.1128/AEM.02095-10

Neutrophilic Iron-Oxidizing "*Zetaproteobacteria*" and Mild Steel Corrosion in Nearshore Marine Environments▽†

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Received 3 September 2010/Accepted 26 November 2010

Microbiologically influenced corrosion (MIC) of mild steel in seawater is an expensive and enduring problem. Little attention has been paid to the role of neutrophilic, lithotrophic, iron-oxidizing bacteria (FeOB) in MIC. The goal of this study was to determine if marine FeOB related to *Mariprofundus* are involved in this process. To examine this, field incubations and laboratory microcosm experiments were conducted. Mild steel samples incubated in nearshore environments were colonized by marine FeOB, as evidenced by the presence of helical iron-encrusted stalks diagnostic of the FeOB *Mariprofundus ferrooxydans*, a member of the candidate class "*Zetaproteobacteria*." Furthermore, *Mariprofundus*-like cells were enriched from MIC biofilms. The presence of *Zetaproteobacteria* was confirmed using a *Zetaproteobacteria*-specific small-subunit (SSU) rRNA gene primer set to amplify sequences related to *M. ferrooxydans* from both enrichments and *in situ* samples of MIC biofilms. Temporal *in situ* incubation studies showed a qualitative increase in stalk distribution on mild steel, suggesting progressive colonization by stalk-forming FeOB. We also isolated a novel FeOB, designated *Mariprofundus* sp. strain GSB2, from an iron oxide mat in a salt marsh. Strain GSB2 enhanced uniform corrosion from mild steel in laboratory microcosm experiments conducted over 4 days. Iron concentrations (including precipitates) in the medium were used as a measure of corrosion. The corrosion in biotic samples (7.4 ± 0.1 mM) was significantly higher than that in abiotic controls (5.0 ± 0.1 mM). These results have important implications for the role of FeOB in corrosion of steel in nearshore and estuarine environments. In addition, this work shows that the global distribution of *Zetaproteobacteria* is far greater than previously thought.

Corrosion of steel is a widely recognized problem that results in major economic costs to industry as well as federal and local governments (23, 31). In the case of microbiologically influenced corrosion (MIC), microbes act to initiate, facilitate, or accelerate electrochemical corrosion reactions (46). Microbes achieve this through their interactions with the environment surrounding the metal surface. For example, bacteria can generate conditions that enhance corrosion through alteration of pH and E_h, excretion of corrosive metabolites, direct or indirect enzymatic reduction or oxidation of corrosion products, formation of biofilms that create corrosive microenvironments, or cathodic depolarization through H_2 metabolism (31). In marine environments, inexpensive mild carbon steel (a steel alloy consisting of ca. 99% Fe, 0.2% C, and 0.8% Mn) is commonly used for construction of ship hulls, steel pilings, pipelines, and other structures; it is protected from corrosion by application of paint or coatings or through cathodic protection (35). Much of the work exploring marine corrosion of mild carbon steel has focused on the activities of sulfate-reducing bacteria and, more recently, Fe(III)-reducing bacteria and methanogens (16). Through corrosion, mild carbon steel is a ready source of $Fe(II)_{(aq)}$ ions and is thus potentially a substrate for growth of aerobic, neutrophilic, chemolithoautotrophic iron-oxidizing bacteria (FeOB) (13). It is therefore surprising that our understanding of the role of FeOB in marine MIC is largely anecdotal. As a metabolic group, the FeOB have been recognized since the 1830s (7a), and in the early 1900s, it was suggested that they might be involved in MIC (3). Only a few subsequent studies have attempted to characterize the influence of FeOB on steel corrosion (e.g., see references 48 and 51). However, these studies were limited by a lack of both appropriate pure cultures of FeOB and an understanding of their biology that could be used to tease out the potential influence of these bacteria on MIC.

Microaerobic, lithotrophic FeOB require redox boundaries where opposing gradients of O_2 and $Fe(II)_{(aq)}$ prevail. This is because the chemical oxidation of $Fe(II)_{(aq)}$ is kinetically hampered at low O_2 concentrations, allowing FeOB to compete (6, 38). In marine habitats, such conditions are known to exist in association with hydrothermal vents, and to date, almost all reports of marine FeOB have been associated with venting, primarily at volcanic seamounts in the deep ocean (5, 7, 13, 15, 17, 21). Cultivation-independent studies have established that a novel candidate class of *Proteobacteria*, the "*Zetaproteobacteria*," tend to be dominant in the iron oxide-rich microbial mats that form at such sites (12). Concurrently, the isolation of a novel FeOB belonging to the *Zetaproteobacteria*, *Mariprofundus ferrooxydans*, from an iron mat at Loihi Seamount, provided phylogenetic confirmation of the uniqueness of *Zetaproteobacteria* (13). *M. ferrooxydans* is an obligate lithotroph whose only known energy source is

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† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 3 December 2010.

TABLE 1. PCR primers designed specifically for and used in this study (see text for details)

Primer name[a]	Sequence (5′–3′)	Target gene and intended specificity
L-C-Zeta-1541-a-S-24	CGA AGT CAG TGA TCC TAT GCT TCC	LSU rRNA gene, specific for *Zetaproteobacteria*
L-C-Zeta-1611-a-A-22	CTC GCC TCG CCT ACC TGT GTC G	LSU rRNA gene, specific for *Zetaproteobacteria*
L-0858-a-S-21	CTA GCC CAT CCA GTG CTC TAC	LSU rRNA gene, broad specificity
L-0858-a-S-21	GTA GAG CAC TGG ATG GGC TAG	LSU rRNA gene, broad specificity
G-C-Zeta-2120-a-A-23	CAG GTG ATT ATG ACC GTG CTG CA	*gyrB* gene, modified to target *Zetaproteobacteria*
G-C-Zeta-1099-a-S-22	CTT GCG ATC ACG CCC CTG TTT G	*gyrB* gene, modified to target *Zetaproteobacteria*

[a] Primers were named according to the Oligonucleotide Probe Database nomenclature scheme. Base position numbers were determined by matching each primer to the sense strand of the corresponding *Escherichia coli* gene; the numbers reflect the *E. coli* 5′-end position number for each primer (1).

$Fe(II)_{(aq)}$ oxidation coupled to respiration of O_2. It excretes a helical stalk comprised of iron oxyhydroxides as it grows. Individual cells can produce hundreds of micrometers of stalk material over time spans of hours to days, an observation that is consistent with microscopic analyses of marine iron mats where stalk-like morphologies are common (4). The goal of this work was to establish if FeOB colonized untreated mild steel exposed to coastal ocean waters and, if they did, to determine whether or not *Zetaproteobacteria* were present.

MATERIALS AND METHODS

Study site descriptions. The isolate described in this study was obtained from a salt marsh on Great Salt Bay, Newcastle, ME (see Fig. S1 in the supplemental material) (44.04169°, −69.53199°). Measured salinities in this brackish marsh range between 0 and 25‰. A persistent iron-oxidizing microbial mat was present in a small gully leading into a tidal creek running through the middle of the marsh. Samples of the mat were taken for microscopy and used as inocula in enrichment cultures. At the time of sampling, the mat temperature was ca. 11.5°C, the porewater pH was 6.2, and $Fe(II)_{(aq)}$ concentrations ranged between 12 and 90 μM.

In situ enrichment experiments were conducted off a dock at Bigelow Laboratory (43.84443°, −69.64095°) in Boothbay Harbor to assess progressive colonization of mild steel coupons by FeOB at the seawater-sediment interface. Samplers were deployed at depths of 5 to 7 m and rested on the surfaces of the sediments. Salinity at this location ranges between 27 and 32‰, and the water temperature ranged from 3 to 10°C over the course of the experiment (29, 34).

Sea samplers were deployed at ca. 60 m in depth 1 to 1.5 km off the coast of Southport Island, ME (43.78°, −69.64°), to assess whether mild steel coupons would be colonized by FeOB at a seawater-sediment interface in deeper water and in the water column. These samplers were designed to rest on the surfaces of the sediments, with additional samplers suspended at ca. 30 m in depth in the water column. Salinities at this location are ca. 32‰, and water temperatures over the course of the incubations ranged from ca. 2 to 15°C at a 50-m depth and from 3 to 21°C at the surface (based on data from http://www.gomoos.org/data/ [buoys D02 and E01]).

Sampler design and deployment. FeOB tend to form very flocculent mats that are loosely adherent; thus, it was necessary to construct special samplers that minimized the disturbance of steel substrata during collection. The samplers were also designed to minimize accumulation of sediment around the steel coupons during incubations on the seafloor and to minimize biofouling of the steel surfaces by algae and invertebrates during long incubations. Two sample designs were used in this study (see Fig. S2 in the supplemental material): polyvinyl chloride (PVC) pipe samplers and PVC pipe samplers containing subsamplers.

The PVC pipe samplers (see Fig. S2A in the supplemental material) were constructed using 1 1/2" Genova PVC pipe coupling, cleanout, and trap fittings (Genova Products Inc., Davison, MI) secured together with vinyl electrical tape (3M). A screen of either 1,000- or 425-μm nylon mesh was inserted into the bottom of the trap fitting to prevent loss of coupons or subsamplers during deployment. Samplers were weighted down with cinder blocks, and the samplers were attached to the retrieval rope with nylon cord or to the cinder blocks with cable ties.

Subsamplers (see Fig. S2B in the supplemental material) were constructed from cutoff 15-ml conical centrifuge tubes (VWR) covered at one end with 90-μm nylon mesh affixed with a silicon tubing band (0.024 OD; VWR) and at the other end with a size 0 black rubber stopper with a hole (VWR). Subsamplers were autoclaved prior to use, and up to three were put in each sampler prior to deployment.

Cold-finish 1018 mild steel coupons and 316L stainless steel control coupons (13 by 15 by 3 mm) were polished with a sheet sander using 220-, 320-, 400-, and 600-grit 3M 413Q Wetordry Tri-M-ite silicon carbide abrasive paper (St. Paul, MN), washed with ethanol and then acetone, dried, weighed, and UV sterilized (UV Stratalinker 2400; Stratagene, La Jolla, CA). Each coupon was transferred aseptically to a sampler or subsampler. Samplers were deployed as soon as possible after coupon preparation.

Isolation and enrichment cultures of iron-oxidizing bacteria. Enrichment and isolation of lithotrophic FeOB were done with environmental samples collected from the Great Salt Bay site or with steel coupons incubated *in situ*. Most enrichments were initiated by serial dilution, using petri plates containing an artificial seawater medium (ASW) and zero-valent iron (ZVI) powder as the source of $Fe(II)_{(aq)}$ [ca. 60 mg of 200-mesh with 99+% Fe(0); Alfa Aesar, Ward Hill, MA]. Details of this method can be found in the work of Emerson and Floyd (11). Plates were incubated at room temperature in a sealed acrylic jar with a BBL Campypak Plus microaerophilic system envelope (Becton, Dickinson and Co., NJ; 5 to 15% O_2). After several days, samples from the plates were checked by phase-contrast light microscopy, and the most dilute plate containing helical iron oxide stalks was selected as the inoculum for further serial dilutions. The enrichments were checked for the presence of heterotrophic bacteria by streaking a sample on ASW-R2A agar plates.

DNA extraction and analysis of phylogenetic genes. To extract DNA from pure cultures of bacteria, we used a Mo Bio PowerSoil kit. DNA from *in situ* samples and enrichment cultures was extracted with a Powerwater DNA isolation kit (Carlsbad, CA). The universal primers 27F (25), 907R (25), 519F (26), and 1492R (47) were used to amplify the small-subunit rRNA (SSU rRNA) gene from the pure-culture DNA extract. For the large-subunit rRNA gene (LSU rRNA), we used the primers 129F, 457R, and 2490R (20). Additional primers were designed based on regions of the gene conserved between these results and the LSU rRNA gene of *Mariprofundus ferrooxydans* PV-1 (NCBI taxonomy ID 314345) and were used to obtain a contiguous LSU rRNA gene sequence; these primers were L-0858-a-A-21, L-0858-a-S-21, L-C-Zeta-1611-a-A-22, and L-C-Zeta-1541-a-S-24 (Table 1). The *gyrB* gene of the isolate was amplified using the universal primers UP-1S and UP-2Sr (49); the primers were modified to match the corresponding sequences of the *gyrB* gene of *M. ferrooxydans* PV-1, and these primers were designated G-C-Zeta-2120-a-A-23 and G-C-Zeta-1099-a-S-22 (Table 1). SSU and LSU rRNA gene primers were checked for specificity using probeCheck (33). Enrichment culture extracts were used to selectively amplify *Zetaproteobacteria* SSU rRNA genes by using the primer combinations 27F and Zeta837R (21) and 1492R and Zeta672F (21). Primers (10 μM [each]) were mixed with AmpliTaq Gold DNA polymerase (Applied Biosystems) and 1 μl of the sample DNA extract and amplified on an Eppendorf Mastercycler personal PCR machine (Hamburg, Germany) under the following conditions: initial denaturation at 94°C for 10 min; 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min; and a final extension of 74°C for 10 min. PCR products were run out on gels, and the bands were excised and extracted with a QIAquick gel extraction kit (Qiagen Inc., Valencia, CA), quantified on a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE), and sent for sequencing analysis at the UIUC Core Sequencing Facility (Urbana, IL). Sequence data for individual genes were constructed into contiguous sequences by using Sequencher (Gene Codes Corp., Ann Arbor, MI) and were compared to sequences of high similarity in GenBank by using the Basic Local Alignment Search Tool (BLAST) (50). Sequences were tested for chimeras by using Bellerophon v. 3 (greengenes.lbl.gov) and Pintail (www.bioinformatics-toolkit.org/Web-Pintail/). Phylogenetic trees were constructed for SSU rRNA gene, LSU rRNA gene, and *gyrB* gene data sequences. Alignments were pre-

pared using CLUSTALW (44), and phylogenetic analyses were conducted in MEGA4 (42). The evolutionary history was inferred using the neighbor-joining method (39), and consensus trees inferred from 1,000 bootstrap replicates were taken to represent the evolutionary history of the taxa analyzed (14). Branches corresponding to partitions reproduced in fewer than 50% of bootstrap replicates were collapsed. All positions containing gaps and missing data were eliminated from the data sets (complete deletion option).

Electron microscopy. Samples were shipped overnight to the Naval Research Laboratory on ice and in sample holders to minimize disturbance to the biofilms. After arrival, the samples were fixed in artificial seawater with 2% (vol/vol) glutaraldehyde buffered with sodium cacodylate (0.1 M, pH 7.2) and stored at 4°C until analysis by environmental scanning electron microscopy–energy-dispersive X-ray spectroscopy (ESEM-EDS) (Electroscan Corp., Wilmington, MA). Coupons were dip rinsed in distilled water to remove fixative and salts, transferred directly to the ESEM, and imaged as described previously (30).

Growth studies of strain GSB2. Strain GSB2, isolated during the course of this work, was tested for the ability to grow on FeS, ZVI, and $FeCl_2$ as sources of $Fe(II)_{(aq)}$, as well as on heterotrophic medium (R2A agarose prepared with ASW medium), according to previously published protocols (11, 13). Salinity tolerance was tested by growing the organism at various mixing ratios of modified Wolfe's mineral medium (MWMM) and ASW medium (11).

To measure the growth of strain GSB2 in opposing gradients of Fe(II) and O_2, gel-stabilized gradient tubes were prepared as detailed by Emerson and Floyd (11), except that ZVI particles were used as a source of $Fe(II)_{(aq)}$ ions in the bottom layer of the tubes. Control tubes containing no source of $Fe(II)_{(aq)}$ were also prepared as a control for heterotrophic growth. Tubes were incubated at 24°C and harvested at 12-h intervals, and cell counts were conducted on the homogenized top layers of agar. Temperature tolerance experiments, also conducted using gel-stabilized gradient tubes with ZVI, were incubated at 7, 15, 20, 24, 31, and 34°C and harvested at 2.5 days.

Laboratory microcosm experiments were prepared to examine corrosion of mild steel coupons incubated with growing cultures of strain GSB2. Samples were prepared in 100-ml Pyrex bottles with open-top caps sealed with polytetrafluoroethylene (PTFE)-faced silicone septa (Corning Inc. Life Sciences, Lowell, MA). A preweighed, UV-sterilized cold-finish 1018 mild steel coupon (75 × 25 × 3 mm), prepared as described for enrichment experiments, was placed in each autoclaved bottle, in addition to 100 ml of sterile ASW medium (11). The medium contained 1 μl ml^{-1} each of MD-TMS mineral and MD-VS vitamin stocks (ATCC, Manassas, VA) and 10 mM bicarbonate and was bubbled with 80:20 N_2:CO_2 for 35 min and with N_2 for 10 min to achieve an initial pH of ca. 7. Triplicate abiotic control bottles were left uninoculated, and triplicate biotic samples were amended with FeOB inoculum (0.5 ml strain GSB2 grown to exponential phase; initial cell density, $1.1 \times 10^5 \pm 0.1 \times 10^5$ cells ml^{-1}). After addition of all materials, each bottle contained 30 ml of ambient air headspace, which equated to approximately 5% O_2 per bottle (vol/vol). Bottles were incubated at 25°C over the course of the experiment. Samples were taken from each bottle at half-day intervals over a 4-day period. At each time point, the bottles were gently inverted three times to homogenize the samples and were sampled for the following parameters: cell counts, $Fe(II)_{(aq)}$, total dissolved iron, $Fe(II)_{(sorbed)}$, $Fe_{(total)}$, oxidation-reduction potential (ORP or E_h), and pH. All geochemical samples were taken after inverting the samples to mix; thus, the samples represent bulk conditions in the sample bottles at each time point. A volume of sterile ASW medium equivalent to that removed was added to each bottle after each sampling. Note that the headspace in these samples was exchanged with normal atmospheric oxygen at each sample point, so about 5% oxygen by volume was added to the samples at each time point.

Determination of cell numbers. Total cell counts of FeOB, most of which adhere to iron oxides, were determined by direct counts under an epifluorescence microscope as previously described (37).

Fe concentration analyses. Analyses of iron concentrations were conducted using methods similar to those described by Lovley and Phillips (32, 41). Briefly, samples for $Fe(II)_{(aq)}$ and total dissolved iron (100 μl) were preserved in 0.45 ml 0.5 N HCl after filtering 200 μl of sample solution through a 4-mm-diameter, 0.22-μm Millex-GV (polyvinylidene difluoride [PVDF]) filter (Millipore, Billerica, MA). Homogenized samples for $Fe_{(sorbed)}$ and $Fe_{(total)}$ analysis (100 μl × 3 replicates per bottle) were also preserved in 0.45 ml 0.5 N HCl. An aliquot of each preserved $Fe(II)_{(aq)}$ and $Fe(II)_{(sorbed)}$ sample (20 μl) was mixed with 0.98 ml of FerroZine reagent solution (Hach, Ames, IA; 1 g $liter^{-1}$ in 50 mM HEPES buffer, pH 7). An aliquot (100 μl) of each $Fe(II)_{(aq)}$ and $Fe_{(total)}$ sample was mixed with 40 μl of 6.25 N hydroxylamine and 60 μl of 0.5 N HCl, incubated for 15 min, and 20 μl of the resulting digest was added to 0.98 ml of FerroZine reagent solution. The various sample-FerroZine mixtures were allowed to develop for 5 min, during which time six 150-μl replicates of each mixture were transferred to a 96-well plate. The sample absorbances were then measured at 562 nm on a Multiskan MCC plate reader (Thermo Electron Corp., Shanghai, China). Fe(II) standards (ranging from 0 to 1 mM) were prepared using $Fe(II)SO_4 \cdot 7H_2O$ dissolved in 0.5 N HCl.

E_h and pH analyses. E_h analyses were conducted on 200-μl aliquots from each bottle by use of an O13 nuclear magnetic resonance (NMR) ORP probe (Sentek, United Kingdom) attached to an Oakton pH 110 m instrument (Vernon Hills, IL). The E_h values were adjusted to standard hydrogen electrode (SHE) values by adding a correction value determined by measuring a standard at each time point (Orion 967961 ORP standard; Thermo Scientific, Beverly, MA). pH analyses were conducted on 200-μl aliquots from each bottle by use of a long-neck pH electrode (Cole-Parmer, Vernon Hills, IL) connected to a Daigger 5500 pH meter (Vernon Hills, IL), calibrated at each time point by using pH 4 and 7 buffers.

Error analyses. Errors were based on standard error calculations for each set of triplicate bottles, with error propagation included in the calculations where additional replicate analyses were performed.

Nucleotide sequence accession numbers. The SSU and LSU rRNA gene and *gyrB* gene sequences for the isolate GSB2 and all other single SSU rRNA gene sequences obtained in this study have been deposited in GenBank under accession numbers HQ206653 to HQ206658.

RESULTS

Isolation of strain GSB. Using an iron-rich microbial mat sample from Great Salt Bay, it was possible to enrich for a stalk-forming FeOB in ASW by using ZVI as the iron source. The original enrichment was diluted to extinction four successive times, with incubations ranging from 6 to 10 days. The culture produced abundant bean-shaped cells that formed twisted iron oxide stalks (Fig. 1A and B). In gradient tube cultures, cell growth presented as a characteristic very sharp band of Fe oxides at the oxide-anoxic interface (Fig. 1C). Growth was not observed in gradient tubes without a source of $Fe(II)_{(aq)}$ or on R2A-ASW plates (incubated both aerobically and in a reduced oxygen atmosphere), indicating the absence of heterotrophic microorganisms. The culture was able to grow by using mild steel as a source of $Fe(II)_{(aq)}$ (Fig. 1F). The SSU and LSU rRNA and *gyrB* genes were sequenced from this culture (GenBank accession numbers HQ206653, HQ206654, and HQ206655, respectively). Compared to the genome of *M. ferrooxydans* strain PV-1 (NCBI taxonomy ID 314345), the SSU rRNA gene was a 96% match over 1,392 bases, the LSU rRNA gene was a 95% match over 2,376 bases, and the *gyrB* gene was an 87% match over 312 amino acids. Phylogenetic trees for these three genes (Fig. 2) illustrate the relationship of GSB2 to PV-1 and confirmed that it is a unique strain in the novel class *Zetaproteobacteria*. It has been designated *Mariprofundus* sp. strain GSB2.

Tests of the salinity tolerance of strain GSB2 indicated that it can survive salinities ranging from 5 to 100% seawater salinity and has an optimal growth temperature of 25°C. It was able to grow on ZVI, FeS, and $FeCl_2$. It did not show evidence of growth in medium containing Mn(II), in R2A-ASW agar, or in low-melting-point agarose without a source of $Fe(II)_{(aq)}$.

Growth on steel. *Mariprofundus* sp. strain GSB2 grew on mild steel coupons in microcosm experiments, and rates of growth were consistent between the replicate samples (Fig. 3A). The calculated doubling time for GSB2 growing on the coupons was between 7 and 8 h. A growth curve for GSB2 grown in diffusion gradient tubes with ZVI as an Fe(II) source yielded a doubling time of 13 h (Fig. 3A). Control gel gradient samples incubated with no source of $Fe(II)_{(aq)}$ did not show an increase in cell number over the course of the experiment (Fig.

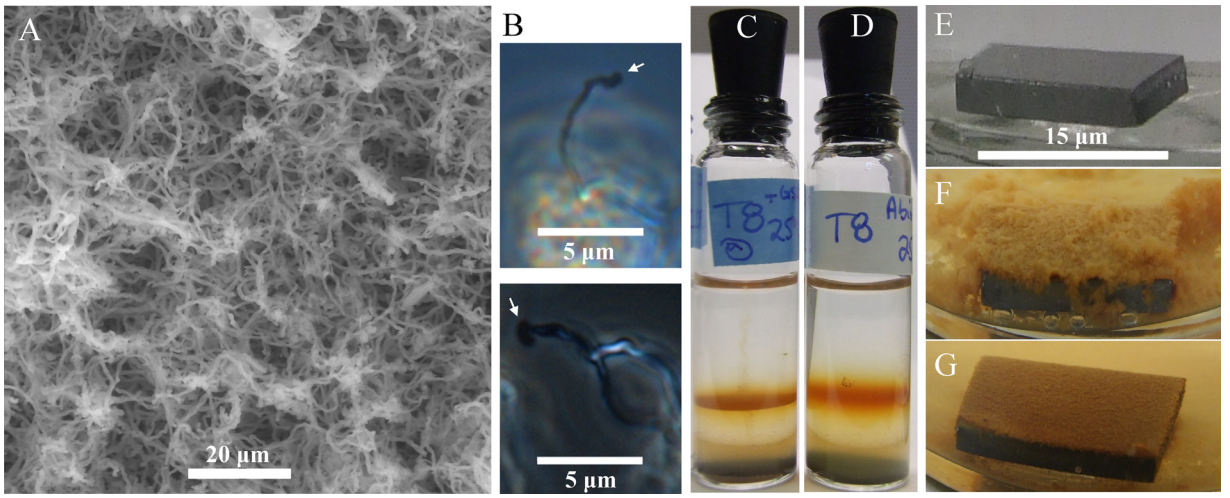

FIG. 1. Overview of morphology of *Mariprofundus* sp. strain GSB2 and growth on metallic iron substrates. (A) ESEM image of helical iron oxide stalks produced by strain GSB2 growing on a mild steel coupon. (B) Phase-contrast image of strain GSB2 cells on stalks (cells indicated with arrows). (C) Strain GSB2 growth in a gradient tube prepared with ZVI as the $Fe(II)_{(aq)}$ source. (D) Abiotic control gradient tube. (E) Uncorroded mild steel coupon. (F) Mild steel coupon with biofilm of strain GSB2 at 4 days. (G) Abiotic corrosion of mild steel coupon at 4 days.

3A). In the case of the gradient tubes containing $Fe(II)_{(aq)}$, a 0.75-ml agarose plug resulted in a diffusion barrier between the bacteria and the $Fe(II)_{(aq)}$ source, which may explain why the growth rate was lower than when the cells were grown directly on the surfaces of steel coupons. Data for $Fe(II)_{(aq)}$, total dissolved iron, Fe(II) sorbed to Fe oxide precipitates, and total iron [$Fe_{(total)}$; including homogenized precipitates] were analyzed to determine if there was a difference in the amounts of iron released from the coupons in the abiotic versus biotic sample incubations. $Fe(II)_{(aq)}$, total dissolved iron, and $Fe(II)_{(sorbed)}$ numbers were very low (<1 mM; maximum at 0.5 day) throughout the experiment (Fig. 3B; see Fig. S3 in the supplemental material). Triplicate $Fe_{(total)}$ samples were taken from each bottle and analyzed to determine whether homogeneity in the samples after mixing was sufficient for replicable results. Results were consistent, and variation between samples was low (Fig. 3B). Total iron released from the coupons over the course of the 4-day experiment was higher for the biotic samples containing strain GSB2 (7.4 ± 0.1 mM) than for the abiotic controls (5.0 ± 0.1 mM) (Fig. 3B), and based on a paired *t* test, the difference was significant ($P < 0.001$). At the completion of the experiment, the submerged portions of the coupons did not appear to have any corrosion product buildup on them or tuberculation, and there was no discernible macroscopic difference in appearance between the surfaces of the coupons used in the abiotic experiments and the coupons incubated with strain GSB2. Qualitatively, the flocculent iron oxides produced by corrosion of the steel surface in the biotic samples were a slightly lighter orange and had a more flocculent appearance than abiotic controls. The pH values in microcosms remained circumneutral and varied between ca. 6.75 and 7.25 (Fig. 3C). There was a slight dip in pH associated with a steep decrease in the E_h values. E_h values for the medium began between 100 and 200 mV and dropped to around −300 mV by day 2.5 (Fig. 3C). The drop in E_h began sooner for the samples containing strain GSB2 and reached a lower E_h value than that of abiotic controls.

***In situ* samplers and enrichments.** Coupons were deployed at the Bigelow dock site in PVC pipe samplers (see Fig. S1 and S2 in the supplemental material). After incubating for 12, 14, and 54 days, the samples were analyzed by ESEM imaging. The characteristic iron oxide-encrusted stalks of FeOB were observed on the coupons at all three time points, and qualitatively, there was an increase in stalks and corrosion products present over the course of the experiment (Fig. 4). Stalks were not observed on a 316L stainless steel control sample harvested at 14 days, and 316L stainless steel controls did not show evidence of corrosion product formation at any of the time points. A biofilm sample of Fe oxides was removed from the surface of a coupon after 68 days of *in situ* incubation and was used to start an enrichment culture. We were unsuccessful in obtaining an FeOB isolate from numerous serial dilutions and transfers of this enrichment; however, the final culture contained stalk-forming FeOB as well as at least one heterotrophic contaminant. When DNA was extracted from one of these mixed cultures and amplified using *Zetaproteobacteria*-specific SSU rRNA gene primers, a single SSU rRNA gene sequence was identified in this sample (Fig. 2, dock D2b-C6 clone [GenBank accession no. HQ206656]).

PVC samplers containing subsamplers were deployed at the site off Southport Island and incubated for periods ranging from 1 to 6.5 months before being harvested (see Fig. S1 and S2 in the supplemental material). SSU rRNA gene sequences were acquired using *Zetaproteobacteria*-specific primers on a DNA extract from a coupon incubated at the sediment surface for 34 days. Remarkably, a single *Zetaproteobacteria* SSU rRNA gene sequence was obtained from this mixed community (Fig. 2, S4-B-H2a clone [GenBank accession no. HQ206658]). Similarly, a single *Zetaproteobacteria* sequence was obtained from an enrichment culture done from a sea sampler coupon

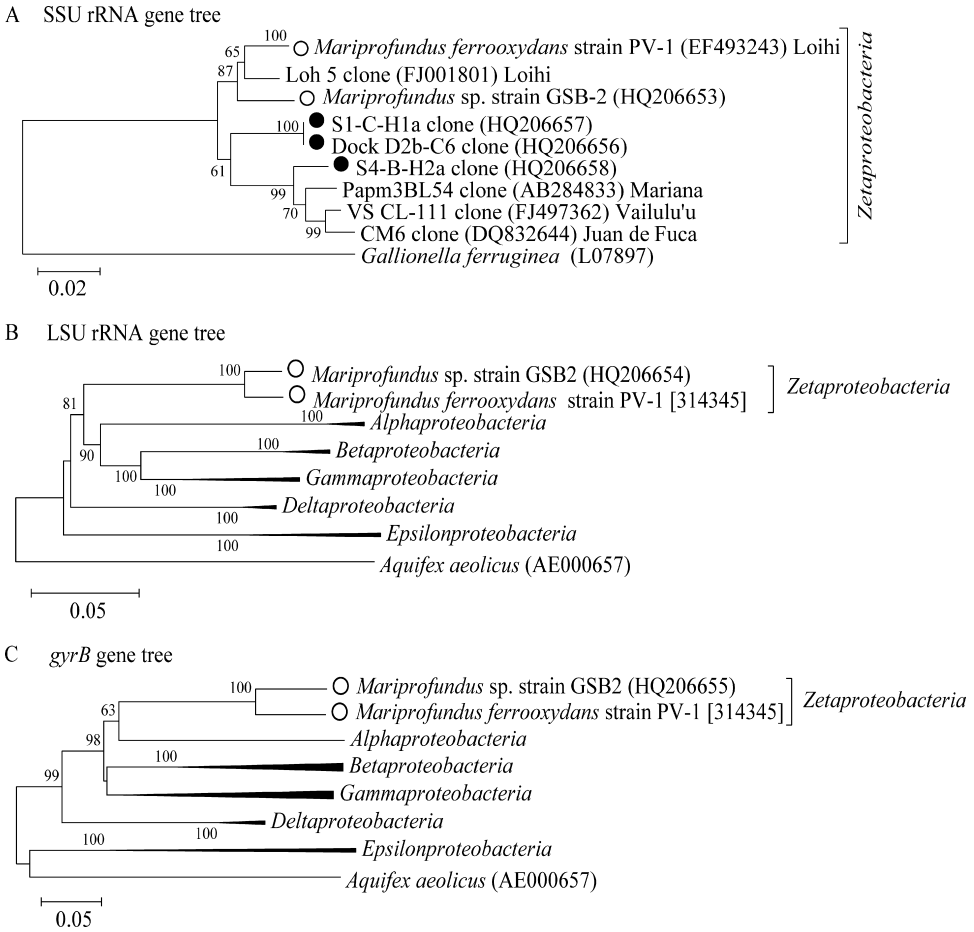

FIG. 2. Neighbor-joining phylogenetic trees showing inferred evolutionary relationships of the isolate and clones detailed in this study relative to other *Zetaproteobacteria* and classes of *Proteobacteria*, based on the SSU rRNA gene (A), the LSU rRNA gene (B), and the *gyrB* gene (C). The tree A outgroup is the iron oxide stalk-forming freshwater organism *Gallionella ferruginea*, and the outgroup for trees B and C is the bacterium *Aquifex aeolicus*. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap tests (1,000 replicates) are shown next to the branches (14). The trees are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic trees. Trees A, B, and C are based on 1,352 positions, 2,091 positions, and 304 positions, respectively, in the final data set. Additional details of tree preparation are provided in the text.

that was incubated in the mid-water column (ca. 30-m depth) for 6.5 months (Fig. 2, S1-C-H1a clone [GenBank accession no. HQ206657]). ESEM images of coupons incubated in the sea samplers also showed evidence of stalk accumulation over the timescale of the incubations (images not shown).

DISCUSSION

Mild steel corrosion and marine FeOB. The work presented here clearly demonstrates that marine FeOB can colonize steel surfaces incubated *in situ* and potentially influence MIC. Previous work on the role of FeOB in MIC of mild steel has not shown the presence of these organisms conclusively or illuminated the role they might play in the process. We have found that FeOB are consistently among the early colonizers on *in situ* coupons during enrichment experiments in nearshore marine environments. Evidence for this includes the presence of the abundant iron oxyhydroxide stalks they characteristically generate, the ready growth of stalk-forming FeOB in enrichment cultures, and amplification of SSU rRNA genes of putative FeOB from corrosion samples. Remarkably, over a 2-month period of *in situ* colonization, the stalks of FeOB dominated the mineral component of corrosion scale biofilms on mild steel coupons (Fig. 4). This implies that marine FeOB are capable of rapid colonization of steel surfaces and can persist for a significant period.

This study also offers evidence that marine neutrophilic FeOB cause enhanced corrosion of mild steel surfaces. To address our original hypothesis that marine FeOB can grow on $Fe(II)_{(aq)}$ released from mild steel, we performed experiments to grow a novel marine FeOB isolate, strain GSB2, on mild carbon steel. This organism was isolated on ZVI and can also grow by using mild steel coupons as a source of $Fe(II)_{(aq)}$ (Fig. 1F and 3). As shown in Fig. 3B, the total amount of iron released from the coupon surface over the 4-day experiment was significantly higher than that released from the abiotic control, and the bacterial cell number increased in concert with this increase in $Fe_{(total)}$ concentration (Fig. 3A).

As yet, the mechanism by which neutrophilic, oxygen-depen-

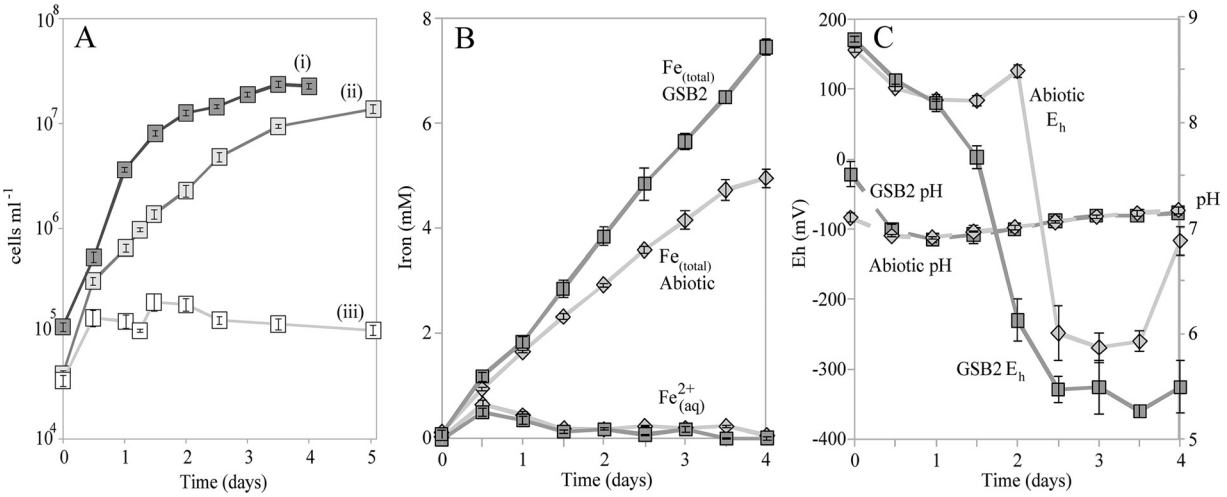

FIG. 3. Corrosion microcosm experiment results for incubations in the presence and absence of *Mariprofundus* sp. strain GSB2. (A) Growth curves for strain GSB2 on coupons (i) and in gradient tubes containing ZVI as the source of $Fe(II)_{(aq)}$ (ii). Note the lack of growth in control gradient tubes containing no source of $Fe(II)_{(aq)}$ (iii). (B) Results for $Fe_{(total)}$ (mM) released from the mild steel coupons and for $Fe(II)_{(aq)}$ in solution. (C) E_h (ORP) and pH data. Standard error bars are present on all samples shown; error bars that are not visible fit within the sample points.

dent FeOB oxidize iron is not understood, nor do we understand how they might enhance the corrosion of steel (10). One general mechanism that has been proposed for bacterial enhancement of corrosion is that extracellular polysaccharides (EPS) released by bacteria can bind and enhance solubilization of $Fe(II)_{(aq)}$ from the metal surface (40). Another possibility is that their requirement and use of $Fe(II)_{(aq)}$ for growth kinetically enhances the release of iron from the steel surface. Perhaps the high chemical reactivity of biologically generated iron oxides relative to that of abiotically produced iron oxides in abiotic controls could also affect Fe(II) release kinetics. Enhanced chemical reactivity of iron oxides has been observed in both naturally occurring freshwater iron mats (27) and lab studies of exopolysaccharides abiotically coprecipitated with iron oxides (36). The iron oxide stalks produced by *Mariprofundus ferrooxydans* have been shown to be composed of a mixture of organic compounds and iron oxides and to have a finer crystallinity than abiotically produced iron oxides (4, 45). Though it has been suggested that FeOB grow passively on steel surfaces and have effects that are limited to enhancing the environment for other, more corrosive colonizers, such as sulfate-reducing bacteria (SRB) (46), it now appears that they

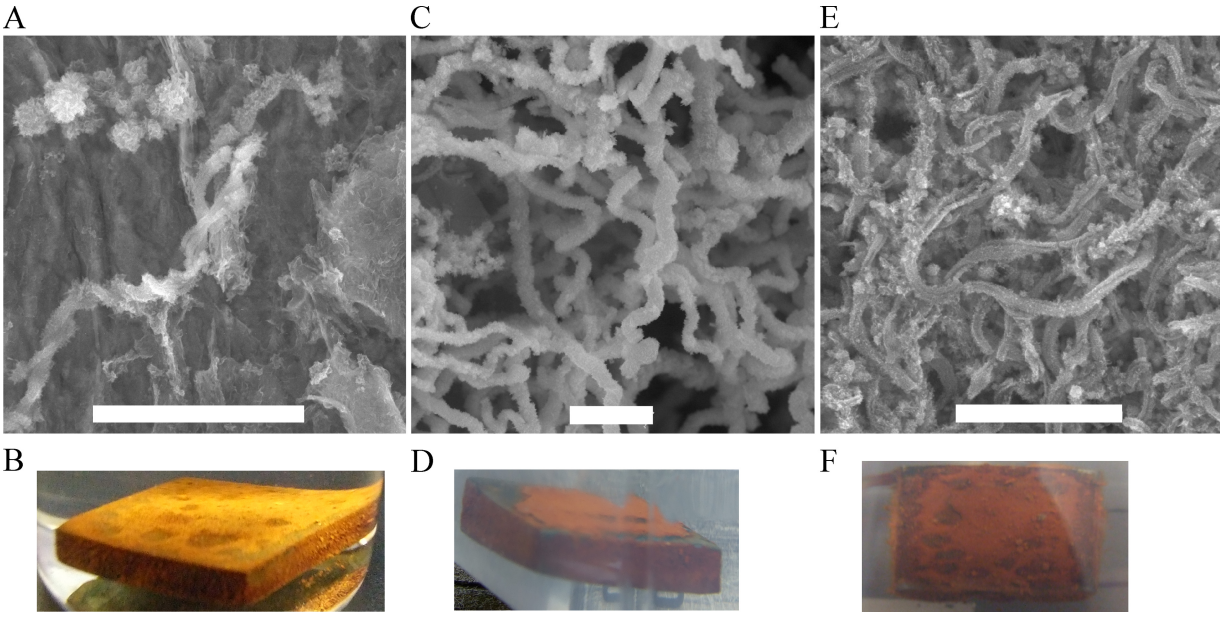

FIG. 4. ESEM and photographic images of *in situ* coupon incubation samples after 12 days (A and B), 14 days (C and D), and 54 days (E and F) of incubation. Note the qualitative increase in helical iron oxide stalks present with time in the ESEM images. Bars on ESEM diagrams (A, C, and E), 10 μm. Coupon (B, D, and F) dimensions are 13 by 15 by 3 mm.

may also have a direct effect on mild steel corrosion. It is also clear that mild steel is an effective substrate for enriching and isolating FeOB of the *Zetaproteobacteria* from nearshore environments.

Our observations on the colonization of mild steel by stalk-forming FeOB have important implications not only for the involvement of FeOB in MIC but also for the more general microbial ecology of mild steel corrosion in marine environments. The sharp decrease in the redox potential we observed in our microcosm experiments suggests that FeOB in association with mild steel corrosion can drive the redox potential down slightly faster than abiotic corrosion processes alone (Fig. 3C). FeOB may thus prime the habitat for anaerobic microbes such as Fe(III)-reducing bacteria (FeRB), SRB, and methanogens to colonize steel surfaces sooner than they would under purely abiotic conditions. Biogenic iron oxides are readily reduced by other bacteria (e.g., FeRB and SRB) in comparison with synthetic iron oxides (2, 8, 28), which may lead to enhanced growth of these anaerobic colonizers. The presence of FeOB-generated stalks in biofilms may also provide a reactive mineral structure to which other bacteria adhere, and thus potentially increase overall bacterial interaction with corrosion products and the steel surface, and provide a framework for developing corrosive microenvironments (31).

Isolation and enrichment of novel FeOB strains and distribution of the *Zetaproteobacteria*. Another significant finding from this work is the discovery of *Zetaproteobacteria* in the coastal ocean. With the isolation of *Mariprofundus* sp. strain GSB2, it is apparent that the distribution of neutrophilic iron-oxidizing *Zetaproteobacteria* is much broader than previously established. Prior studies have shown that *Zetaproteobacteria* are found primarily at Fe-rich deep-sea vents that are most often associated with seamounts (9). The one exception where sequences of *Zetaproteobacteria* have been found in a shallow water hydrothermal setting is at the Santorini volcano in Greece. This is a site with waters that are high in $Fe(II)_{(aq)}$ and where copious mats of FeOB develop. Previous studies that were based primarily on morphological identification suggested that the stalk-forming FeOB *Gallionella* sp. was responsible for formation of these mats (18, 19); however, a recent molecular survey of Santorini found *Mariprofundus*-like sequences but not *Gallionella* sequences (17). This is consistent with the observation that *Gallionella* and related FeOB of the *Betaproteobacteria* are found in freshwater environments, while FeOB related to the *Zetaproteobacteria* are marine (10). However, in coastal environments not influenced by hydrothermal venting, to our knowledge, there has been only one report of a single *Zetaproteobacteria* sequence found in sediments associated with a fish farm in Wakasa Bay, Japan (22), and no *Mariprofundus*-like FeOB have been isolated. The isolation of *Mariprofundus* sp. strain GSB2 represents the first FeOB of the *Zetaproteobacteria* isolated from a coastal habitat. The facts that it forms a stalk, appears to be an obligate lithotrophic FeOB, and shares 96% similarity with *M. ferrooxydans* based on the SSU rRNA gene suggest that it may belong to a new species in the genus *Mariprofundus*. Amplification of DNA from enrichment cultures also confirmed that these contained *Zetaproteobacteria*, but these organisms were less closely related to *M. ferrooxydans* than strain GSB2. One surprising finding was that a single *Zetaproteobacteria* sequence was obtained from at least one of the *in situ* samplers, suggesting that a single clone may have been responsible for establishment and colonization of the steel surface. We are presently working to establish whether all putative *Zetaproteobacteria* present on *in situ* enrichment coupons are associated with stalks and/or involved in iron oxidation.

That *Zetaproteobacteria* appear to quite rapidly colonize steel surfaces in the coastal ocean suggests that there may be a reservoir of FeOB that are poised to quickly take advantage of sources of Fe(II) when they become available, e.g., exposed steel surfaces. Our observation that mats containing FeOB can exist in discrete patches in a salt marsh suggests one such reservoir. However, it is also possible that they exist more widely in marine sediments that have an abundant distribution of iron sulfides. These ecosystems may be dominated by sulfur cycling bacteria; however, it has also been shown that iron reduction can play a significant role in some marine sediments (43). If iron cycling is rapid and tightly coupled in these environments, there might be little visible evidence (i.e., iron oxide deposits) for iron oxide precipitation. Thus, the potential exists for marine sediments to support smaller populations of FeOB. The fact that these organisms have largely gone undetected in coastal sediments indicates that their overall population levels are probably low; nonetheless, these cells could provide the seed for a rapidly propagating population once an abundant source of reduced iron is present.

ACKNOWLEDGMENTS

Funding for this project was provided by the U.S. Office of Naval Research (award number N0001410WX20247).

We acknowledge the help of Matthew Parkhurst for deployment and harvesting of sea samplers.

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